Accelerated diabetic glomerulopathy in galectin-3/AGE receptor 3 knockout mice

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ABSTRACT **Several molecules were shown to bind advanced glycation end products (AGEs) in vitro, but it is not known whether they all serve as AGE receptors and which functional role they play in vivo. We investigated the role of galectin-3, a multifunctional lectin with (anti)adhesive and growth-regulating properties, as an AGE receptor and its contribution to the development of diabetic glomerular disease, using a knockout mouse model. Galectin-3 knockout mice obtained by gene ablation and the corresponding wild-type mice were rendered diabetic with streptozotocin and killed 4 months later, together with age-matched nondiabetic controls. Despite a comparable degree of metabolic derangement, galectin-3-deficient mice developed accelerated glomerulopathy vs. the wild-type animals, as evidenced by the more pronounced increase in proteinuria, extracellular matrix gene expression, and mesangial expansion. This was associated with a more marked renal/glomerular AGE accumulation, indicating it was attributable to the lack of galectin-3 AGE receptor function. The galectin-3-deficient genotype was associated with reduced expression of receptors implicated in AGE removal (macrophage scavenger receptor A and AGE-R1) and increased expression of those mediating cell activation (RAGE and AGE-R2). These results show that the galectin-3-regulated AGE receptor pathway is operating in vivo and protects toward AGE-induced tissue injury in contrast to that through RAGE.—Pugliese, G., Pricci, F., Iacobini, C., Leto, G., Amadio, L., Barsotti, P., Frigeri L., Hsu, D. K., Vlassara, H., Liu, F.-T., Di Mario, U. Accelerated diabetic glomerulopathy in galectin-3/AGE receptor 3 knockout mice. *FASEB J.* 15, 2471–2479 (2001)**

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ENHANCED FORMATION OF advanced glycation end products (AGEs) resulting from nonenzymatic glycation seems to play a major role in the abnormal glomerular remodeling underlying diabetic glomerular disease (1). In fact, AGEs have been shown to promote mesangial extracellular matrix (ECM) deposition (2) via AGE receptor-mediated up-regulation of various cytokines (3, 4). The AGE receptor-mediated pathway is also involved in the uptake and degradation of irreversibly glycated molecules, thus favoring their removal from tissues and circulation (1).

Several AGE binding proteins have been identified, including OST-48 (AGE-R1), 80K-H (AGE-R2), galectin-3 (AGE-R3), RAGE, the macrophage scavenger receptor A (MSR-A), particularly type II, lactoferrin, and lysozyme (1). Despite the great number of molecules shown to bind AGEs in vitro, few data are available concerning their ability to serve as AGE receptors in vivo. This extreme heterogeneity might also imply binding and/or functional specificity, with these receptors binding different AGE structures and/or playing distinct functional roles. RAGE is known to mediate AGE-induced cell activation via induction of oxidative stress and stimulation of p21(ras)-dependent mitogen-activated protein kinase (MAPK) (5) and its downstream targets NF-κB (6) and the AP-1 complex (5), whereas the MSR-A seems to be involved in AGE uptake and degradation (7). AGE-R1, AGE-R2, and galectin-3/AGE-R3 are thought to behave as an AGE receptor complex (8), with AGE-R1 implicated predominantly in AGE removal and AGE-R2 participating in cell activation.

Galectin-3, the most recently identified AGE binding protein, is a member of the lectin family interacting with the β-galactoside residue of several cell surface and ECM glycoproteins via the carbohydrate recognition domain (9). It is also capable of peptide–peptide associations mediated mainly by its unique short NH_2 terminus (9). These structural properties enable galectin-3 to exert multiple functions, including *1)* regula-

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tion of cell-to-matrix (laminin) interactions, with a predominant anti-adhesive effect (10); *2)* activation of mast cells via binding to IgE and the IgE receptor (11); *3)* regulation of gene expression due to its pre-mRNA splicing activity (12); *4)* control of cell proliferation and death, the latter possibly related to sequence homology and association with *bcl-2* (13); and *5)* high-affinity binding, internalization, and degradation of AGEs (8, 14). We recently reported that galectin-3 is not expressed at the glomerular/mesangial level under normal conditions, but becomes expressed with aging, and that the diabetic milieu induces/enhances its expression, which occurs earlier and to a greater extent than during normal aging (15). This (over)expression of galectin-3 might participate in the pathogenesis of diabetic glomerular disease by virtue of its AGE receptor and growth-regulating properties, both potentially influencing the process of glomerular remodeling.

This study was aimed at evaluating the in vivo relevance of galectin-3 as an AGE receptor and its functional role in favoring the removal of AGEs and/or mediating the effects of these adducts in terms of cell activation and induction of tissue injury. We investigated the effects of galectin-3 deficiency on the development of experimental diabetic glomerulopathy, by using galectin-3 knockout (KO) mice, obtained by gene ablation, which were rendered diabetic with streptozotocin and killed 4 months later, together with the corresponding wild-type (WT) animals.

MATERIALS AND METHODS

Experimental design

Adult (12 wk) male galectin-$3^{-/-}$ (KO) and galectin-$3^{+/+}$ (WT) mice were divided into the following groups (n=14): *1)* nondiabetic WT; *2)* nondiabetic KO; *3)* diabetic WT; and *4)* diabetic KO. Mice were rendered diabetic by a single intraperitoneal injection of 180 mg/kg body weight streptozotocin (Sigma Chemical Co., St. Louis, MO). Of the 14 nondiabetic animals per each genotype, 10 were used for the assessment of all parameters of kidney function and structure, and 4 for glomeruli isolation and extraction of RNA from them. Of the 14 diabetic mice per each genotype, one died before the study ended, whereas 4 animals did not become hyperglycemic or spontaneously reversed their diabetes, thus serving as controls for the effect of streptozotocin independent of hyperglycemia. The animals received water and food ad libitum. When needed, diabetic animals were given supportive insulin treatment (Ultratard, Novo Nordisk, Denmark; 1 IU/kg body weight twice a week) in order to prevent ketosis without affecting significantly blood glucose levels. Metabolic control was assessed by measuring body weight and blood glucose at regular intervals as well as glycated hemoglobin (Hb) levels in blood samples obtained at the end of the study. Renal function was assessed by measuring serum creatinine levels and the total protein/creatinine and albumin/creatinine ratios in urine samples collected at the time of death. Renal structure was assessed by evaluating morphology at histological examination and measuring kidney wet weight, mean glomerular area and mesangial fractional area, together with kidney cortex mRNA levels for the ECM components fibronectin, laminin B1 and collagen IV α1 chain and the prosclerotic cytokine TGF-β1. The role of growth-regulating actions of galectin-3 was evaluated by measuring glomerular cell proliferation and death rates. To assess whether the differential renal outcome in diabetic KO vs. WT mice was related to the lack of galectin-3 AGE receptor function, the circulating and renal AGE levels and renal/glomerular AGE receptor expression were also evaluated.

Generation of galectin-3-deficient mice

Galectin-3-deficient animals were generated from C57BL/6 mice by producing a targeted disruption of the galectin-3 gene in mouse embryonic stem cells, as previously reported (16). Homozygous mice resulting were viable and fertile; when compared with galectin-3 expressing mice, they exhibited comparable reproductive capacities and similar findings with respect to blood chemistries, peripheral blood leukocyte and erythrocyte counts, lymphocyte subpopulations, and histological analyses of organs and tissues (16).

Assessment of metabolic control

Body weights were measured twice a week and served as a guide for supportive insulin treatment. Blood glucose levels were measured weekly by Hemoglukotest 20–800 strips (Boehringer Mannheim, Milan, Italy) with the aid of an automated colorimetric instrument (Reflolux® II M, Boehringer Mannheim) from a drop of blood obtained by tail venipuncture. Glycated Hb levels were assessed by boronate affinity gel chromatography using the Glyco-Test™ II 100 (Pierce Chemical, Rockford, IL), as previously reported (17).

Assessment of renal function

Renal filtration function was evaluated by measuring serum creatinine levels by the Jaffe method (17). Glomerular barrier function was determined by assessing total protein and albumin concentrations in urine samples obtained at the time of death. Total protein levels were assessed by using the Bradford method and urinary albumin concentrations were measured with ELISA (17). In this assay, a polyclonal rabbit anti-rat albumin antibody (Cappel, Cooper Biomedical, Malvern, PA) was coated onto a 96-well plate (Nunc, Roskilde, Denmark) at 4°C overnight. Urine samples or standard mouse albumin (Sigma) were then added, followed by the same anti-rat albumin antibody conjugated with peroxidase (Cappel) and the peroxidase substrate to reveal the reaction. Results were normalized to the urine creatinine levels, as measured by the Jaffe method, and expressed as total protein/creatinine and albumin/creatinine ratio, respectively.

Assessment of renal structure

Kidneys were quickly removed from the animals under anesthesia, cleaned of the surrounding fat, washed in sterile saline solution, and weighed. A sagittal section of the right kidney was immediately fixed by immersion in phosphate-buffered 4% paraformaldehyde solution and routinely embedded in paraffin; the remaining tissue was frozen in liquid nitrogen for AGE and protein measurements. Sections were cut, stained with hematoxylin-eosin, periodic acid Schiff, and periodic acid-silver methenamine (PASM). For morphometric analysis, at least 60 glomerular tuft profiles per sample, selected on PASM stained sections by moving from external to deep cortex in a serpentine manner (with exclusion of profiles containing <3 mesangial tracts), were analyzed with the aid of a computer image analysis system. The areas of each glomerular tuft profile and areas of each glomerulus

occupied by the mesangium, as identified by dense PASM staining, were measured. The mean glomerular area and the mean mesangial area for each animal were then obtained together with the mesangial fractional area, calculated by the formula [(mean mesangial area/mean glomerular area) × 100] (18). Evaluation of tubulo-interstitial damage was performed by one of us (P.B.) blinded to the group assignment by the use of a semiquantitative scale (0 to 4) for grading the extension of lesions in two nonconsecutive whole kidney sections per animal.

Assessment of glomerular cell proliferation and death

PCNA expression and DNA fragmentation were detected in paraffin-embedded sections as indexes of cell proliferation and death, respectively. PCNA expression was assessed immunohistochemically using a mouse monoclonal antibody (PC10) raised against human PCNA and cross-reacting with the mouse antigen (Dako, Glostrup, Denmark). Immunostaining was performed for 1 h at room temperature (RT) in 0.5% BSA at 1:50 dilution. To avoid nonspecific staining of tissue proteins by the secondary anti-mouse IgG antibody, the mouse monoclonal antibody was revealed with HistoMouse kit (Zymed Laboratories, San Francisco, CA) and the secondary biotinylated antibody with a streptavidin-fluorescein conjugate (Dako) after blocking of tissue biotin. DNA fragmentation was assessed by the terminal deoxynucleotidyl transferase (TdT) -mediated d-UTP-biotin nick-end labeling (TUNEL) method, using a fluorescein-based kit obtained from Boehringer Mannheim.

Assessment of ECM, TGF-β1, and AGE receptor gene expression

Transcripts for fibronectin were measured by Northern blot analysis, as described previously (17), whereas those for laminin B1, collagen IV α1 chain, TGF-β1, AGE-R1, AGE-R2, galectin-3/AGE-R3, RAGE, and MSR-A type II were quantified by competitive RT-PCR (19, 20). Renal cortex from the left kidney was separated from medulla, then total RNA was extracted by the guanidine thiocyanate-phenol-chloroform method. RNA was also extracted by the same method from glomeruli isolated by standard sieving techniques from four nondiabetic mice of each genotype. The purity of RNA preparation was confirmed by an absorbance 260:280 ratio >1.9. For Northern blot analysis, total RNA (30–40 μg/lane) was electrophoresed under denaturing conditions in 1.2% agarose (Life Technologies, Gaithersburg, MD) gel, blotted onto Hybond-N nylon membranes (Amersham, Amersham, UK), and U.V. fixed. Filters were prehybridized in Rapid-Hyb Buffer (Amersham) for 15 min at 65°C, then hybridized for 2 h at 65°C in the same solution containing 25 ng of cDNA labeled using the Prime-IT™ II Random Primer Labeling Kit (Stratagene, La Jolla, CA) and 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) as precursor. The 500 bp *Eco* RI rat FN cDNA and the 2200 bp *Bam* H1 human β-actin cDNA were used as probes (17). Filters were sequentially washed with SSC 2×, SSC 1×, and SSC 0.1× + 0.1% SDS for 15 min at 65°C, then exposed to Hyperfilm-MP autoradiography films (Amersham) with the aid of intensifying screens at −70°C for 1–7 days. Quantification of mRNA levels was performed by scanning densitometry using a Gel Doc 2000 Densitometer equipped with the Quantity One software (Bio-Rad Laboratories, Hercules, CA). To account for differences in sample loading, results were normalized to the β-actin signal. For competitive RT-PCR, 1 μg of total RNA was reverse transcribed using Retroscript kit (Ambion, Austin, TX). The following primers were used: laminin B1 sense 5′ CAA GCT TGA GAG AGG AAC GTG G 3′-antisense 5′ TTA CCT TGG TCA CCG AGC 3′; collagen IV α1 chain sense 5′ TAG GTG TCA GCA ATT AGG CAG G 3′-antisense 5′ TCA CTT CAA GCA TAG TGG TCC G 3′; TGF-β1 sense 5′ ATA CAG GGC TTT CGA TTC AGC 3′-antisense 5′ GTC CAG GCT CCA AAT ATA GG 3′; AGE-R1 sense 5′ GCT CTT CCA CTC CTT ACT CCT 3′-antisense 5′ CCA GAC AGG CAA CTA TGA AC 3′; AGE-R2 sense 5′ TGG TGT GGT GGC TAT TGA CCT TTG C 3′-antisense 5′ CGC AGT AGT CGT CGT TCA CCT GAT C 3′; AGE-R3/galectin-3 sense 5′ CAC CTG CAC CTG GAG TCT AC 3′-antisense 5′ GCA CTG GTG AGG TCT ATG TC 3′; RAGE sense 5′ CCT GGG AAG CCA GAA ATT 3′-antisense 5′ GCA CAG GTC AAG GTC ACA 3′; MSR-A type II sense 5′ AGA CCT TCT GTC GTT CCC CT 3′-antisense 5′ CCC TGG GAC AGT GTT CTC TGG TT 3′; and β-actin (for normalization) sense 5′ TCT AGG CAC CAA GGT GTG 3′-antisense 5′ TCA TGA GGT AGT CCG TCA GG 3′. The mutants were made by creating a deletion in the original PCR product. Competitive PCR was performed using six different amounts of mutant based on preliminary experiments performed to establish the range of mutant concentrations producing a slope of the line close to one and within which the equivalence point falls (approximately in the middle). After electrophoresis of PCR products, the ratio of unknown cDNA/mutant was quantified by scanning densitometry and results were expressed as the ratio of each AGE receptor to the β-actin mRNA level.

Assessment of renal AGE receptor protein expression

Protein expression for the AGE receptors AGE-R1, AGE-R2, and RAGE was evaluated by Western blot analysis of total (for the latter two) or plasma membrane (for AGE-R1) extracts of kidney cortex obtained from frozen tissue samples. Tissue was cut into small pieces, the medulla was discarded, and the remaining material was homogenized using 3 ml/g tissue of buffer containing 150 mM NaCl, 50 mM Tris-HCl, 1% v/v Tween 20, 0,5% Na-deoxycholate, 0.1% SDS, 10 mM PMSF, 9 μg/ml leupeptin, 6 μg/ml aprotinin, and 2 μg/ml pepstatin, pH 8.0. The homogenate was centrifuged at 15,000 *g* for 30 min at 4°C and the supernatant was collected. To obtain plasma membrane extracts, kidney cortex samples were homogenized in ice-cold PBS + protease inhibitors and centrifuged three times at 500 *g* for 5 min at 4°C to remove contaminating blood. The pellet was centrifuged at 500 *g* for 15 min at 4°C in a buffer consisting of PBS containing 200 mM HEPES, 1 mM EDTA, 30 mM KCl, 100 mM $MgCl_2$, 2 mM PMSF, 10 mM benzamidine, 2 mM DTT, 25 μg/ml leupeptin, and 6 μg/ml aprotinin to pellet unbroken cells and nuclei, then the supernatant was centrifuged at 40,000 *g* for 2 h at 4°C to collect the membrane fraction. Finally, this fraction was washed three times in the extraction buffer and resuspended in a lysis buffer containing 0.1% SDS and Nonidet P-40 Tergitol + protease inhibitors. For Western analysis (17), protein samples (10–30 μg) were added with an equal volume of sample buffer 2× (100 mM Tris-HCl, pH 7.4, 5% SDS, 10% saccharose, 1 mM Na_2 EDTA, 0.025% bromphenol blue, 0,1 M dithiothreitol), separated by SDS-PAGE (9–10% of 30% Acrylamide/Bis solution, 29:1), and transferred by electroblotting using a MINI PROTEAN II™ (Bio-Rad Laboratories) onto PVDF membranes (Amersham). The membranes were incubated overnight at 4°C under agitation with TBS-Tween (TBS+0.5% Tween 20) + 5% nonfat dry milk (NFDM) to block the nonspecific reactivity, then probed for 1 h at RT under agitation with the primary antibody diluted in TBS-Tween + 3% NFDM. Antisera against AGE-R1 and AGE-R2 produced in rabbit using purified recombinant OST-48 and 80K-H proteins (21), diluted 1:500 and 1:250, respectively, and a rabbit polyclonal antibody against recom-

binant human RAGE (kindly provided by Dr. A. McKay, University of L'Aquila, Italy) at a concentration of 5 μg/ml were used. Subsequently, the membranes were incubated for 45 min at RT with an appropriate IgG conjugated with peroxidase (Dako), diluted 1:1,500 in TBS-Tween + 3% NFDM, washed, and developed with ECL reagent (Amersham). Immunocomplexes were revealed by autoradiography and quantitated by scanning densitometry. To quantify RAGE expression, the ~35 kDa (truncated form), 45 kDa (full-length protein), and 65 kDa (glycosylated form) bands were analyzed (22). Results were normalized to the signal of β-actin, revealed by use of a goat polyclonal antibody raised against the carboxyl terminus of human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000.

Assessment of circulating and renal AGE levels

The level of AGEs in plasma and kidney cortex extracts obtained from frozen tissue samples was assessed by a competitive ELISA technique (23), using a monoclonal antibody raised against BSA-AGE that recognizes at least carboxymethillysine (24), a RAGE ligand shown to activate cell signaling pathways and modulate gene expression (25). Renal AGE levels were also assessed immunohistochemically in paraffin-embedded sections by a mouse monoclonal antibody anti-methylglyoxal (26) used at 1:200 dilution and revealed with HistoMouse SP kit, as described above, and a rabbit polyclonal antibody raised against RNase-AGE, shown to recognize specific in vivo-formed AGEs of uncertain structure (23), used at 1:100 dilution and revealed by a swine anti-rabbit IgG conjugated with fluorescein (Dako). Results for glomerular AGE accumulation were expressed as percent of glomeruli showing a positivity at the peripheral or mesangial level, calculated on 60 glomerular tuft profiles. Tubular AGE accumulation was also evaluated semiquantitatively.

Statistical analysis

Values are expressed as mean ± SD; the percent change in diabetic animals vs. nondiabetic controls was also calculated. Statistical significance was evaluated by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. All statistical tests were performed on raw data.

RESULTS

Metabolic control

Metabolic derangement and growth impairment were similar in the diabetic KO and WT mice (**Table 1**). Nonfasting blood glucose and glycated Hb levels were ~four- and twofold higher in diabetic than in nondiabetic mice of both genotypes, corresponding to values detected in poorly controlled diabetic patients. During the 4-month period of the study, body weights increased much less in diabetic than in nondiabetic mice (+10% vs. +35%) after an initial loss following the injection of streptozotocin.

Renal function

Serum creatinine levels did not significantly differ among the experimental groups (**Fig. 1**). Glomerular barrier function was significantly impaired in both diabetic groups, with more pronounced changes in the diabetic KO vs. WT mice (Fig. 1). The total protein/creatinine ratio increased by 64% in KO and 74% in WT diabetic mice; the albumin/creatinine ratio by 141% in KO and 203% in WT diabetic mice. Mice receiving streptozotocin, but not developing hyperglycemia or reversing their diabetes, did not show increased protein excretion (not shown).

Renal structure

At histological examination (**Fig. 2**), renal tissue from nondiabetic KO and WT mice had a normal appearance. In the diabetic mice, glomerular lesions were more pronounced in KO than WT mice, whereas diffuse tubulo-interstitial damage (mean score>2) was observed in one diabetic WT and three diabetic KO animals. Kidney weight was increased in diabetic vs. nondiabetic animals, with no significant difference between diabetic KO and WT mice (Fig. 1). Computer assisted measurements revealed significantly enlarged glomerular areas and mesangial fractional areas in diabetic kidneys from both genotypes, with more marked increases vs. the corresponding nondiabetic animals detected in KO (+19% and+60%, respectively) vs. WT (+10% and +16%, respectively) mice (Fig. 1). The difference in mesangial fractional area between the two diabetic groups (+52% in KO vs. WT mice) was highly significant and much more pronounced than the nonsignificant increase (+10%)

TABLE 1. *Final body weight, nonfasting blood glucose and glycated hemoglobin (Hb) levels, and circulating and renal/glomerular AGE levels in nondiabetic (ND) and diabetic (D) WT and KO mice (mean±SD; n in parentheses)*

	WT-ND	KO-ND	WT-D	KO-D
Final body weight (g)	30.8 ± 1.8 (10)	30.7 ± 1.6 (10)	24.5 ± 1.5 (9)*	24.3 ± 1.1 (9)*
Nonfasting blood glucose (mmol/l)	5.5 ± 0.3 (10)	5.5 ± 0.3 (10)	22.6 ± 2.1 (9)*	22.8 ± 1.8 (9)*
Glycated Hb (%)	6.6 ± 0.6 (10)	6.6 ± 0.6 (10)	14.1 ± 1.1 (9)*	14.2 ± 1.3 (9)*
Circulating AGEs (AGE U/ml serum)	1.8 ± 0.6 (4)	2.1 ± 1.1 (4)	5.2 ± 0.9 (4)#	7.4 ± 1.2 (4)*¶
Renal AGEs (AGE U/mg tissue)	4.2 ± 0.7 (4)	4.4 ± 0.8 (4)	14.0 ± 1.5 (4)#	26.5 ± 2.6 (4)*¶
Glomerular AGEs‡ (% of positive glomeruli)				
Peripheral	2.1 ± 1.2 (4)	3.0 ± 0.7 (4)	43.9 ± 28.2 (5)*	65.7 ± 6.6 (5)*†
Mesangial	0 (4)	0 (4)	9.0 ± 3.2 (5)*	23.0 ± 7.6 (5)*¶

Significantly different from the corresponding ND mice at * $P < 0.001$ or # $P < 0.01$. Significantly different in KO-D vs. WT-D mice at ¶ $P < 0.001$ or † $P < 0.01$. ‡ Data obtained with the anti-methylglioxal monoclonal antibody (see text).

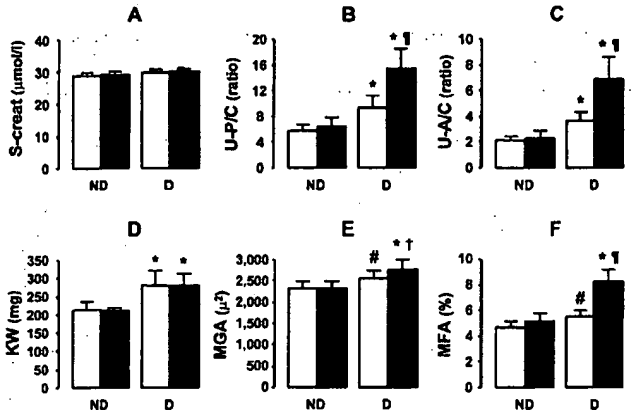

Figure 1. Renal functional and structural parameters. Serum creatinine (S-creat, *A*), urinary protein/creatinine (U-P/C, *B*) and albumin/creatinine (U-A/C, *C*) ratios, kidney weights (KW, *D*), mean glomerular areas (MGA, *E*), and mesangial fractional areas (MFA, *F*) in nondiabetic (ND) and diabetic (D) WT (□) and KO (■) (mean±SD; $n=10$ for ND and 9 for D mice). Significantly different from the corresponding ND mice at $^{*}P < 0.001$ or $^{\#}P < 0.05$; in KO-D vs. WT-D mice at $^{\P}P < 0.001$ or $^{\dagger}P < 0.05$.

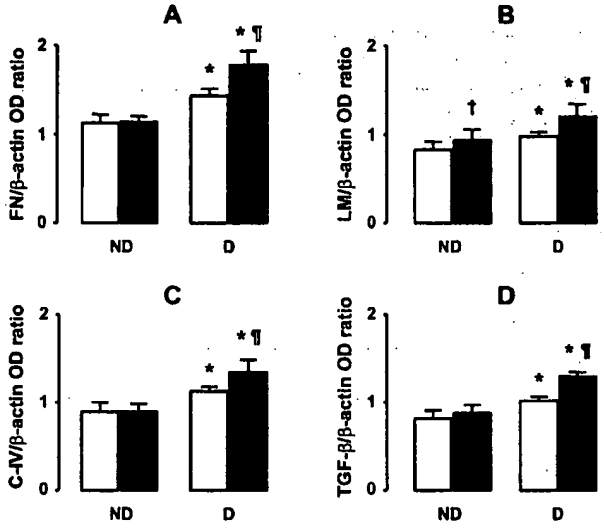

Figure 3. Renal cortex ECM gene expression. Renal cortex mRNA levels for fibronectin (FN, *A*), laminin B1 (LM, *B*), collagen IV α1 chain (C-IV, *C*), and TGF-β1 (TGF-β *D*) (expressed as OD ratio to β-actin mRNA level) in nondiabetic (ND) and diabetic (D) WT (□) and KO (■) mice (mean±SD; $n=8$ per group). Significantly different from the corresponding ND mice at $^{*}P < 0.001$; in KO-D vs. WT-D mice at $^{\P}P < 0.001$; in KO-ND vs. WT-ND mice at $^{\dagger}P < 0.05$.

observed in the nondiabetic KO vs. WT mice (Fig. 1). Again, no renal damage was observed in mice receiving streptozotocin but not developing hyperglycemia or reversing their diabetes (not shown).

Renal ECM and TGF-β1 gene expression

No change in the kidney cortex mRNA levels for fibronectin, collagen IV α1 chain, or TGF-β1 was detected between the two nondiabetic groups, whereas laminin B1 gene expression was higher in KO than in WT control mice (+13%) (**Fig. 3**). Transcripts for ECM components and TGF-β increased in both genotypes in response to diabetes, with changes vs. the corresponding nondiabetic controls more pronounced in KO (+29–56%) than in WT (+19–28%) diabetic mice (Fig. 3).

Glomerular cell proliferation and death

Glomerular expression of PCNA was negligible and unchanged in response to diabetes in both KO and WT mice, with a low labeling index (data not shown). Likewise, TUNEL-positive cells were very rare in the glomeruli of both KO and WT mice and their number was virtually unaffected by diabetes; tubular staining was more evident, with a slight increase observed in the

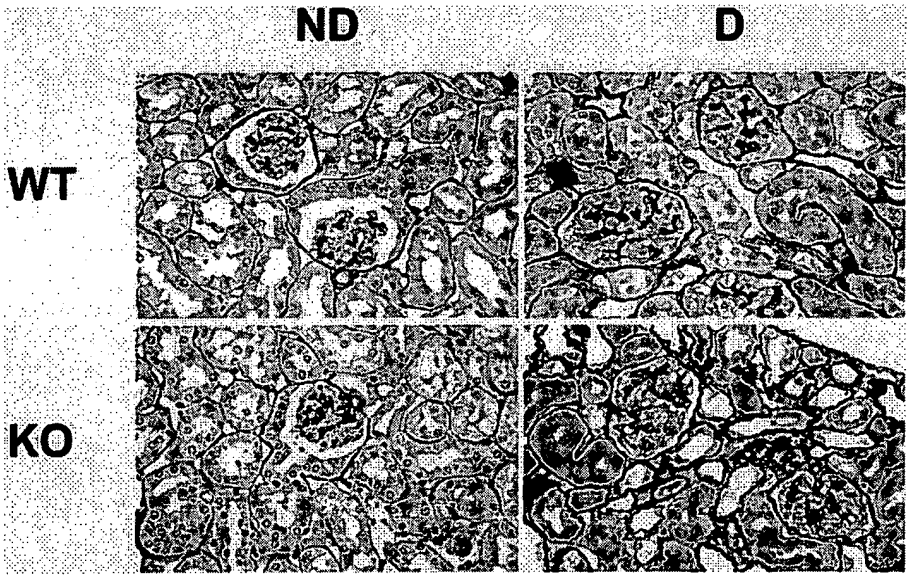

Figure 2. Renal morphology. Histological appearance of kidney sections from nondiabetic (ND) and diabetic (D) WT and KO mice (PASM, 100×).

diabetic vs. nondiabetic KO mice but no change between the diabetic and nondiabetic WT animals (not shown).

Circulating and renal AGE levels

The serum levels of AGEs were similar in the two nondiabetic groups and increased with diabetes in both genotypes, though values were 42% higher in diabetic KO than WT mice (Table 1). The kidney content of AGEs, as assessed by ELISA, was increased 3.3-fold in the diabetic WT mice and 6-fold in the diabetic KO mice vs. the corresponding nondiabetic animals (Table 1). Anti-AGE-positive sites were detected in renal tissue from all experimental groups. In nondiabetic WT and KO mice, glomeruli showed no or slight peripheral, possibly epithelial, staining. In diabetic mice, many glomeruli showed peripheral AGE immunoreactivity and a smaller percentage exhibited a positivity within the mesangial areas. Both peripheral and mesangial staining was more pronounced in diabetic KO vs. WT animals (Table 1). In all animals, most of the AGEs was localized in tubular cells; in the diabetic mice from both genotypes, staining was more evident in the inner cortex and medulla (not shown).

Renal AGE receptor mRNA and protein expression

Nondiabetic KO mice showed a reduced content of AGE-R1 (−30%) and MSR-A type II (−11%) and increased levels of AGE-R2 (+44%) and RAGE (+52%) mRNA in the renal cortex (**Fig. 4**). Analysis of glomerular gene expression for these receptors confirmed this pattern, with a less marked reduction in AGE-R1 (−9%, P=NS) and a more pronounced reduction in MSR-A type II (−19%, $P<0.01$) mRNA levels (not shown). Protein expression of AGE-R2 (particularly RAGE) was increased and that of AGE-R1 reduced in the renal cortex of nondiabetic KO vs. WT animals (**Fig. 5**). Renal cortex AGE receptor mRNA levels were also affected by diabetes. In the diabetic WT mice, AGE-R1 was reduced vs. the nondiabetic WT mice by 32% whereas AGE-R2 was increased by 33%, galectin-3/AGE-R3 by 51%, RAGE by 63%, and MSR-A type II by 93%. In the diabetic KO mice, the increases vs. controls in AGE-R2 (+46%) and RAGE (+85%) were more marked; those in MSR-A type II (+54%) were less evident than those detected in the diabetic WT animals. Moreover, diabetic KO mice showed a less pronounced reduction in AGE-R1 vs. the nondiabetic controls (−16%) than the diabetic WT (−32%), but their absolute mRNA levels for this receptor were significantly lower than those detected in the nondiabetic WT animals (−41%) due to the reduced expression of AGE-R1 related to the genotype (Fig. 4).

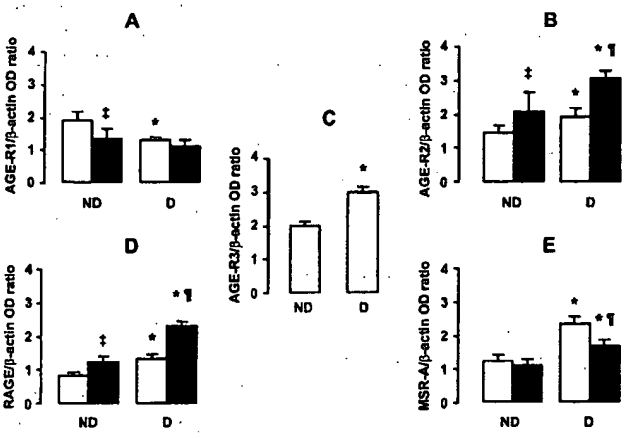

Figure 4. Renal cortex AGE receptor gene expression. Renal cortex mRNA levels for AGE-R1 (*A*), AGE-R2 (*B*), AGE-R3 (*C*), RAGE (*D*), and macrophage scavenger receptor A type II (MSR-A, *E*) (expressed as OD ratio to β-actin mRNA level) in nondiabetic (ND) and diabetic (D) WT (□) and KO (■) mice (mean±SD; n=8 per group). Significantly different from the corresponding ND mice at $^{*}P < 0.001$; in KO-D vs. WT-D mice at $^{¶}P < 0.001$; in KO-ND vs. WT-ND mice at $^{‡}P < 0.001$.

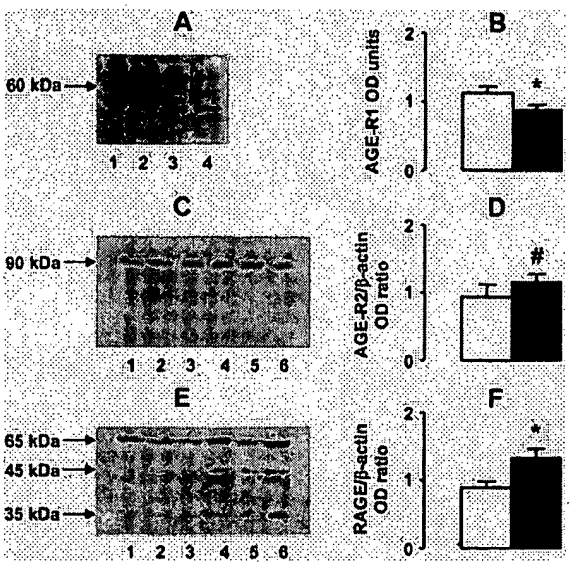

Figure 5. Renal cortex AGE receptor protein expression. Renal cortex protein levels for AGE-R1 (*A, B*), AGE-R2 (*C, D*) and RAGE (*E, F*) in nondiabetic (ND) WT (□) and KO (■) mice. Left: representative Western blots from selected animals (*A*: lanes 1 and 2=membrane and cytosolic fractions from a WT-ND, lanes 3 and 4=membrane and cytosolic fractions from a KO-ND; *C, E*) lanes 1–3=WT-ND, lanes 4–6=KO-ND). Right: densitometric analysis of protein expression (*B*: OD units; *D, F*: OD ratio to β-actin signal; mean±SD; n=6 per group). Significantly different in KO-ND vs. WT-ND mice at $^{*}P < 0.001$ or $^{\#}P < 0.05$

DISCUSSION

The concurrent assessment of renal functional and structural alterations associated with diabetes in KO and WT animals indicated that galectin-3-deficient mice develop accelerated glomerulopathy, despite a degree of metabolic derangement comparable to that

observed in mice expressing galectin-3. Diabetic glomerulopathy in the diabetic KO mice was associated with more pronounced increases in the circulating and particularly renal/glomerular AGE levels vs. the diabetic WT animals, whereas the rate of cell proliferation and death was unchanged. In addition, nondiabetic KO mice showed complex alterations in the mRNA and protein expression of the other AGE receptors at the renal/glomerular level vs. the corresponding WT animals; changes in response to diabetes were also different in the two genotypes.

Both diabetic KO and diabetic WT mice showed renal functional and structural changes attesting to the development of diabetic glomerular disease, in keeping with previous observations in experimental diabetic animals (17, 27). These alterations include increases in urinary total protein/creatinine and albumin/creatinine ratios, kidney wet weight, mean glomerular area, mesangial fractional area, and scoring of tubulo-interstitial lesions. As previously reported by our group and other investigators (17, 27–29), experimental diabetic glomerulopathy was associated with increased gene expression of the ECM components fibronectin, laminin, and collagen IV and an unchanged rate of glomerular cell proliferation and apoptosis, thus confirming the predominance of matrix vs. cell alterations in the pathogenesis of mesangial expansion occurring in diabetes (30), at variance with other glomerular diseases in which enhanced glomerular cell replication and/or death seem to play a major role.

An intriguing finding in this study was that most changes were significantly more pronounced in the diabetic KO than WT animals, with the latter group showing a mild form of glomerulopathy, in keeping with previous observations in rodents with experimental diabetes of 4 month duration (17). This was evidenced by the twofold increase in proteinuria and fourfold greater increment in mesangial expansion, the most prominent functional and structural features of diabetic glomerular disease (30), detected in diabetic KO vs. WT mice. These differences in the severity of renal involvement between the two diabetic groups occurred despite similar degrees of metabolic derangement, as evidenced by the comparable extent of increases in blood glucose and glycated Hb levels and impairment in body growth. This observation pointed to a link between galectin-3 deficiency and the development of accelerated diabetic glomerulopathy.

Whether the enhanced susceptibility to renal injury induced by diabetes in the KO mice was related to the lack of galectin-3 AGE receptor function or of other actions of this multifunctional molecule cannot be conclusively established by these experiments. The unchanged rates of glomerular cell proliferation and apoptosis detected in the KO vs. WT mice seem to argue against the hypothesis that cell cycle-regulating properties of galectin-3 were involved in the accelerated glomerulopathy. On the contrary, the finding that renal cortex mRNA levels of laminin, the main extracellular galectin-3 ligand (9), but not of fibronectin and collagen IV, were increased in the nondiabetic KO vs. WT mice suggests that the (anti)adhesive function of galectin-3 may be implicated in favoring excess matrix accumulation in mice lacking this molecule. However, the more pronounced AGE accumulation detected in serum and kidneys of diabetic KO vs. WT mice, despite similar degrees of metabolic derangement and glycated hemoglobin levels, strongly suggests that the accelerated diabetic glomerulopathy observed in the KO mice was attributable to an impaired AGE removal from extrarenal tissues and at the renal/glomerular level. Hence, more AGEs accumulated within the renal tissues of diabetic KO mice, thus producing (together with an increased activation of glomerular/mesangial cells in response to them) the more marked up-regulation of matrix gene expression and mesangial expansion observed in these animals.

Taken together, these results provide the first experimental evidence that galectin-3 plays a significant role as an AGE receptor in vivo, as previously shown for RAGE (31) and suggested for MSR-A (32) and AGE-R1 (33). Another major advancement in the knowledge about AGE receptors is the demonstration that these molecules may exert different functions, with the galectin-3-regulated pathway serving as a protective mechanism toward AGE-induced injury in contrast to that of RAGE, which appears to promote target tissue damage through the induction of oxidative stress (34). This relevant, protective role of galectin-3 may depend on an interference with RAGE signaling and/or transduction of AGE signals through yet unknown intracellular pathways distinct from MAPK activation and induction of oxidative stress, possibly mediating AGE internalization and degradation (8, 14). This hypothesis is in contrast with the lack of a transmembrane anchor sequence or signal peptide in galectin-3 structure (9), suggesting that it acts in association with other AGE receptor molecules rather than as an independent AGE receptor. Alternatively, galectin-3 may influence the expression of other components of the AGE receptor complex and of other AGE binding proteins, thus regulating the overall AGE receptor function. This interpretation is strongly supported by the observation that nondiabetic KO mice exhibited reduced renal/glomerular levels of AGE-R1 and MSR-A and an increased content of AGE-R2 and RAGE vs. nondiabetic WT mice, providing evidence that these receptors, though structurally unrelated, exhibit a coordinate regulation that can be explained solely by their common AGE receptor function. As a consequence, galectin-3 deficiency may have acted through complex changes in the pattern of expression of the other AGE receptors. This rearrangement reduced the ability of the cell to remove AGEs (via the decrease of MSR-A and AGE-R1) and favored AGE receptor-mediated cell activation (through the increase of RAGE and AGE-R2). This altered expression pattern of AGE receptors did not appear to cause tissue damage at physiological AGE levels, but could have resulted in accelerated glomerular disease in the presence of increased amounts of

circulating and/or tissue AGEs formed during chronic hyperglycemia. The different AGE receptor changes in response to diabetes could also be responsible for the differential course of glomerulopathy in the two genotypes through a greater increase in RAGE and AGE-R2, as well as a less pronounced up-regulation of MSR-A and a further reduction in AGE-R1 expression.

The mechanism by which galectin-3 deficiency induced such an altered expression pattern of the other AGE receptors might reside in the complex and yet unknown interactions with them. Previous evidence indicated that galectin-3 is physically associated with AGE-R2, since it was isolated by screening an expression library from activated macrophages using an anti-AGE-R2 antibody (8), and colocalizes with both AGE-R1 and AGE-R2 to caveolae (35). The sequence homologies of Mac-2 (galectin-3) binding protein with MSR (36) and the involvement of 80K-H (homologous to AGE-R2) in the activation of MAPK pathway participating in RAGE signaling (31) suggest that galectin-3 may also directly or indirectly interact with these two AGE receptors.

In conclusion, these experiments showed that galectin-3 deficiency resulted in accelerated diabetic glomerulopathy. This finding was associated with marked renal/glomerular AGE accumulation and a modified expression pattern of the other AGE receptors linked to the genotype, suggesting that it was attributable to the lack of galectin-3 AGE receptor function. These results provided the first experimental evidence that galectin-3 plays a significant role as an AGE receptor regulator in vivo and that this AGE receptor-regulated pathway functions as a protective mechanism toward AGE-induced tissue injury, in contrast to that of RAGE. Galectin-3 KO mice may represent a useful animal model for the study of the pathogenesis of diabetes- (and AGE-) induced glomerular disease. FJ

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